Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Potency Testing of *Pasteurella multocida* Bacterins of Porcine Origin

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes procedures for potency testing biological products containing *Pasteurella multocida* of porcine origin, as prescribed in the Code of Federal Regulations, Title 9 (9 CFR), Part 113.121. Mice are vaccinated twice, 14 days apart, and challenged with a standard dose of virulent *P. multocida* 10-12 days after the second vaccination.

1.2 Keywords

Pasteurella multocida; porcine; mouse; potency; vaccination-challenge; 9 CFR, Part 113.121; bacterin

2. Materials

2.1 Equipment/instrumentation

2.1.1 Spectrophotometer, Spectronic 70™ (Bausch and Lomb, Rochester, New York) or equivalent

2.1.2 Bunsen burner

2.1.3 Incubator, 37°C

2.1.4 Micropipettors, 20-200 µl and 200-1000 µl

2.1.5 Vortex mixer

2.1.6 Crimper for aluminum caps on serum vials

2.2 Reagents/supplies

2.2.1 *P. multocida* challenge culture, IRP PPC-serial 2, strain 169. This culture is available from the Center for Veterinary Biologics-Laboratory (CVB-L),

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Ames, Iowa.

2.2.2 Test bacterin(s) containing *P. multocida*

2.2.3 APHIS-approved *P. multocida* reference bacterin, PMA169 serial 3. This reference bacterin expires on **January 1, 2003**. It is available from the CVB-L, USDA-APHIS, Ames, IA.

2.2.4 Syringes, 1 ml

2.2.5 Needles, 26 ga, 3/8 in

2.2.6 Glass serum bottle, 20 to 100 ml

2.2.7 Rubber stopper, 13 x 20 mm, and aluminum cap for serum bottle

2.2.8 Glass screw-top tubes, 13 x 100 mm, with caps, or equivalent tubes to fit spectrophotometer

2.2.9 Pipettes, 5 ml, 10 ml, 25 ml

2.2.10 Micropipette tips, up to 1000 µl capacity

2.2.11 Blood agar plates

2.2.12 Tryptose broth

2.2.13 Phosphate-buffered saline (PBS)

2.2.14 Water, distilled or deionized, or water of equivalent purity

2.3 Animals

2.3.1 Mice, 16-22 g (Although the 9 CFR does not specify a specific mouse type, the CVB-L uses CF-1 mice.)

2.3.2 Sixty mice are required for each bacterin to be tested (20 mice/dilution x 3 dilutions/bacterin). Sixty additional mice are required for the reference bacterin. Thirty mice are required to determine the LD_{50} of the challenge inoculum. All mice must be from the same source colony and of similar weight and/or age.

3. Preparation for the test

3.1 Personnel qualifications/training

Technical personnel must have a working knowledge of the use of general laboratory chemicals, equipment, and glassware and must have specific training and experience in sterile technique, the handling of live bacterial cultures, and the handling of mice.

3.2 Selection and handling of test animals

3.2.1 Mice of either sex may be used, but females are recommended.

3.2.2 All mice must be housed and fed in a similar manner.

3.2.3 Identify each cage of mice by treatment group.

3.2.4 If any mice die after vaccination, but prior to challenge with live *P. multocida*, perform a necropsy on these mice to determine cause of death if the cause of death is not outwardly apparent. If the cause of death is unrelated to vaccination, file the necropsy report with the test records, and no additional action is needed. If death is attributable to the test bacterin, report the death immediately to the Center for Veterinary Biologics-Inspection and Compliance (CVB-IC), which may request further safety testing of the bacterin.

3.2.5 When the test is concluded, instruct the animal caretakers to euthanize and incinerate the mice and to sanitize contaminated rooms.

3.3 Preparation of supplies/equipment

3.3.1 Sterilize all glassware before use.

3.3.2 Use only sterile supplies (pipettes, syringes, needles, rubber stoppers, diluents, etc.).

3.3.3 All equipment must be operated according to manufacturers' instructions and maintained and calibrated, as applicable, according to current CVB-L Standard Operating Procedures (SOPs).

3.4 Preparation of reagents

3.4.1 Pasteurella Multocida Bacterin. Reference bacterin PMA169, Serial 3. Expiration date **January 1, 2003.** Dilute this reference bacterin 1:2 in PBS (i.e., equal parts bacterin and diluent) immediately prior to use. (For purposes of calculating PD_{50}, the 1:2 dilution is considered undiluted when comparisons are made with the test bacterin.) After making the initial 1:2 dilution, make 3 fivefold dilutions of bacterin in PBS. Place each of the fivefold dilutions in separate sterile injection vials.

3.4.2 Test bacterin(s) containing *P. multocida*. For each test bacterin, make 3 fivefold dilutions in PBS immediately prior to use. Place each of the dilutions in separate sterile injection vials. Retain original vial(s) of bacterin to use undiluted.

3.4.3 *P. multocida* challenge culture. The challenge culture, IRP PPC-serial 2, strain 169 is lyophilized in 2-ml amounts. Store vials of lyophilized culture at $\leq$4°C.

3.4.4 Phosphate-buffered saline (NVSL Media 10559)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Sodium phosphate, dibasic	1.15 g
Potassium phosphate, monobasic	0.2 g
Water	q.s. to 1000 ml

Adjust pH to 7.2 ± 0.1. Autoclave 20 min at 121°C. Store at 20°-25°C for no longer than 6 mo.

3.4.5 Tryptose broth (NVSL Media #10404)

Tryptose broth powder	26 g
Water	q.s. to 1000 ml

Autoclave 20 min at 121°C. Cool before using. Store at 20°-25°C no longer than 6 mo.

3.4.6 Bovine blood agar (NVSL Media #10006)

Blood agar base powder	40 g
Water	q.s to 950 ml

Autoclave 20 min at 121°C. Cool to 45°-47°C. Add 50 ml defibrinated bovine blood. Pour into sterile petri dishes. Allow to cool to room temperature. Store at 2°-7°C for no longer than 6 mo.

4. Performance of the test

4.1 Vaccination of test animals

4.1.1 Check the label on each product to confirm identity and dose volume.

4.1.2 Test each test bacterin and the reference bacterin at 3 fivefold dilutions. Typically, test the bacterins undiluted, 1:5 and 1:25. **Reminder: Make an initial 1:2 dilution of IRP PMA 169 Serial 3 to create "undiluted" reference bacterin for purposes of this**

assay. It is permissible to make fivefold dilutions other than those described as long as the reference and test bacterins are tested at the same dilutions. For viscous bacterins, it is advisable to start at 1:2 or 1:3 and make fivefold dilutions from this starting point to increase injectability of the product at the low dilution.

4.1.3 Thoroughly mix product by inverting end-to-end at least 10 times. Make the appropriate fivefold dilutions of the reference bacterin in PBS. Make identical fivefold dilutions of the test bacterin(s) in PBS or the diluent approved in the specific outline of production for that product. (Some oil-adjuvanted products require oil-based diluents.) Place each dilution in a separate sterile injection vial. Prepare dilutions immediately prior to use; do not store in diluted form.

4.1.4 Weigh 5 randomly selected mice immediately prior to vaccination to assure that the average body weight of the mice is between 16 and 22 g. Record weights.

4.1.5 Vaccinate separate groups of 20 mice with each of the 3 test bacterin dilutions and 3 reference bacterin dilutions. For reference bacterin groups, inject each mouse with 0.1 ml intraperitoneally. Inject test bacterins intraperitoneally at a dose volume that corresponds to 1/20 of the least dose recommended on the product label. This volume must not be <0.1 ml. (For products with a minimum dose volume of <2 ml, dilute as needed in approved diluent to create a 2-ml-dose volume, so that 1/20 dose is 0.1 ml.)

Note: It is permissible to vaccinate a few extra mice in each group to compensate for any potential deaths that may occur prior to challenge and are not related to vaccination. However, if extra mice are vaccinated, all surviving at the time of challenge must be challenged with live *P. multocida* and included in data calculations.

4.1.6 Revaccinate the mice in a similar manner 14 days after the first vaccination.

4.1.7 Retain 30 nonvaccinated mice to determine LD_{50} of the challenge.

4.2 Preparation of challenge

4.2.1 Reconstitute a vial of challenge in 1 ml tryptose broth.

4.2.2 Inoculate 4 blood agar plates with a loopful of reconstituted culture and streak for isolation.

4.2.3 Incubate the inoculated blood agar plates at 36°-38°C for 16-18 hr.

4.2.4 Use plates that have pure growth by visual inspection to prepare the challenge inoculum.

4.2.5 Scrape several bacterial colonies from the surface of the blood agar plates and suspend in tryptose broth in a 13 x 100-mm tube. Add bacterial growth until the suspension measures 70% ± 2% T at 630 nm using a Spectronic 70 spectrophotometer.

Note: Use sterile tryptose broth in a 13 x 100-mm tube as a blank for the spectrophotometer.

4.2.6 Prepare a 10^{-5} dilution of the standardized culture in tryptose broth. **This is the inoculum used to challenge the mice and is hereinafter referred to as "challenge inoculum."** Prepare a minimum of 25 ml of challenge inoculum to inoculate 120 mice. Place in a serum vial and seal with a rubber stopper and aluminum ring. Save an aliquot(s) of this inoculum in a separate vial(s); retain vial(s) as a sample for postchallenge plate counts.

4.2.7 Make additional tenfold dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of the challenge inoculum to determine LD_{50} of the challenge. Place each dilution in a separate labeled serum vial and seal.

4.2.8 Place all vials of challenge on ice to transport to animal room. Keep on ice through challenge procedure and until culture is added to plates for postinoculation plate count.

4.3 Timing and administration of challenge

4.3.1 Challenge all vaccinates 10-12 days after the second vaccination.

4.3.2 Challenge nonvaccinated LD_{50} controls at the same time as the vaccinates.

4.3.3 Inoculate each vaccinated mouse with 0.2 ml of challenge inoculum intraperitoneally, using a 26-ga, 3/8-in needle.

4.3.4 Inoculate separate groups of 10 nonvaccinated control mice intraperitoneally with 0.2 ml of each of the LD_{50} dilutions.

4.4 Postinoculation plate count

4.4.1 After mice are challenged, perform a colony count on blood agar plates according to the current version of BBSOP0019, using the vials retained for this purpose.

4.4.1.1 Use tryptose broth as the diluent for the plate count, and plate on blood agar. Incubate aerobically at 36°-38°C for 18-30 hr.

4.4.1.2 Calculate the colony-forming units (cfu) per challenge dose according to the following formula:

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Note: Average count in 0.1 ml culture x 2 x dilution factor (see table below)=cfu/0.2-ml dose of challenge culture

If plates used for avg. count were inoculated with:	Dilution factor
10^{-3} dilution of challenge inoculum	1000
10^{-4} dilution of challenge inoculum	10000
10^{-5} dilution of challenge inoculum	100000

4.5 Observation of mice after challenge

4.5.1 Observe the mice daily for 10 days after challenge. Record deaths.

4.5.2 If deaths occurring after challenge are suspected to be due to causes other than pasteurellosis, perform a necropsy on such mice to determine the cause of death. If cause of death is unrelated to vaccination and/or challenge, do not include the deaths in the total deaths for the test.

5. Interpretation of the test results

5.1 Interpret the test as prescribed in 9 CFR, Part 113.121.

5.1.1 Calculate the LD_{50} (theoretical dose/dilution at which the challenge would be lethal to 50% of the control mice) of the challenge inoculum using the Reed-Muench or Spearman-Karber method of estimation. A valid test must have an LD_{50} between 100 and 10,000.

5.1.2 Calculate the PD_{50} of the reference bacterin (theoretical dose/dilution at which the bacterin would

protect 50% of the mice) using the Reed-Muench or Spearman-Karber method of estimation.

If the PD_{50} of the reference bacterin cannot be calculated because the lowest dilution protects <50% of the mice or the highest dilution protects >50% of the mice, the test is invalid. The reference bacterin also must protect >0% and <100% of the mice at 2 or more dilutions in a valid test.

5.1.3 Calculate the PD_{50} of each test bacterin in a manner identical to that used in **Section 5.1.2**.

5.1.3.1 If the PD_{50} of the test bacterin cannot be calculated because the lowest dilution tested protects <50% of the mice, the bacterin may be retested, ***provided that*** the following conditions are met:

1. If the bacterin is not retested, it is unsatisfactory.

2. If the protection provided by the lowest dilution of the reference exceeds that provided by the lowest dilution of the test bacterin by at least 6 mice, the test bacterin is unsatisfactory without additional testing.

3. If the total number of mice protected by the reference (sum of survivors in all dilution groups) exceeds the total number protected by the test bacterin by 8 mice or more, the test bacterin is unsatisfactory without additional testing.

5.1.3.2 If the PD_{50} of the test bacterin in a valid test cannot be calculated because the highest dilution protected more than 50% of the mice, the test bacterin is satisfactory without further testing.

5.1.4 Divide the PD_{50} of each test bacterin by the PD_{50} of the reference to calculate the Relative Potency (RP) for each test bacterin.

5.1.5 If the RP of the test bacterin(s) is <0.50, the test bacterin is unsatisfactory.

5.1.5.1 A test bacterin with an RP <0.50 may be retested by conducting 2 independent replicate tests in a manner identical to the initial test. Calculate the results of the retests in the following manner:

1. Average the RP values of the retests.

2. If the average RP of the retests is <0.50, the bacterin is unsatisfactory.

3. If the average RP of the retests is >0.50 **AND** the RP obtained in the original test is 1/3 or less than the average (RP) of the retests, the test bacterin is satisfactory. Consider the initial test to be the result of test system error.

4. If the average of the retests is >0.50 **BUT** the RP of the original test is greater than 1/3 of the average RP of the retests, calculate a new average RP using the RP values obtained in all tests (original plus retests). If the new average RP is >0.50, the test bacterin is satisfactory. If the new average RP is <0.50, the test bacterin is unsatisfactory.

5.1.6 Record the plate count (cfu/dose) of the challenge on the test result form. This information is for informational purposes to track trends and to troubleshoot problem tests. The 9 CFR does not specify a minimum or maximum cfu/dose for this test.

6. Report of test results

Report the results of the test(s) as described by the current version of BBSOP0020.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.121, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Reed LJ, Muench H, 1938. A simple method of estimating 50% endpoints. *Am J Hygiene*, 27:493-497.